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Steering the osteoclast through the demineralization–collagenolysis balance ☆

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ABSTRACT

There is a lot of interest for how and how much osteoclasts resorb bone. However, little is known about the mechanism which controls the orientation and the duration of a resorptive event, thereby determining the specific geometry of a cavitation. Here we show that the relative rate of collagenolysis vs. demineralization plays a critical role in this process.

First we observed that when culturing osteoclasts on bone slices, excavations appeared either as round pits containing demineralized collagen, or as elongated trenches without demineralized collagen. This suggests that round pits are generated when collagen degradation is slower than demineralization, and trenches when collagen degradation is as fast as demineralization. Next we treated the osteoclasts with a low dose of a carbonic anhydrase inhibitor to slightly decrease the rate of demineralization, thereby allowing collagen degradation to proceed as fast as demineralization. This resulted in about a two-fold increase of the proportion of trenches, thus supporting our hypothesis. The same result was obtained if facilitating collagen degradation by pre-treating the bone slices with NaOCl. In contrast, when decreasing the rate of collagenolysis vs. demineralization by the addition of a cathepsin K specific inhibitor, the proportion of trenches fell close to 0%, and furthermore the round pits became almost half as deep. These observations lead to a model where the osteoclast resorption route starts perpendicularly to the bone surface, forming a pit, and continues parallel to the bone surface, forming a trench. Importantly, we show that the progress of the osteoclast along this route depends on the balance between the rate of collagenolysis and demineralization. We propose that the osteocytes and bone lining cells surrounding the osteoclast may act on this balance to steer the osteoclast resorptive activity in order to give the excavations a specific shape.

Introduction

Bone resorption is critical to model and remodel the skeleton during growth and adult life, and may also lead to pathological bone destruction and fragilization. Bone resorption is performed by OCs,[1] specialized cells able to solubilize both of the two main bone constituents, mineral and collagen. Mineral is solubilized by protons generated by carbonic anhydrase and pumped into the resorption lacunae. This exposes the collagen fibers which become available for degradation by proteinases [1]. CatK, a cysteine proteinase, has been identified as the major proteinase responsible for the degradation of collagen by OCs [1–3].

The magnitude of bone resorption and the control of this magnitude have been a major trigger in research on OCs. Accordingly, a diversity of tools were developed in order to quantify bone mass and bone resorption levels in the clinic and in preclinical models, and clinical treatments were designed to reduce these resorption levels. However, bone shaping during growth does not depend only on how much bone is resorbed but also on where it is resorbed. Similarly, fracture risk does not result only from decreased amount of bone, but also from changes in bone structure. Spacing, distribution, connectivity, and shape of trabeculae all contribute to bone strength, and are features affected by hormones like glucocorticoids, estrogen, or PTH, which are also known to affect bone strength [4–8]. Of note, these changes in architecture result from the sum of individual resorption events, and are therefore likely to be influenced by the geometry of the individual OC resorption lacunae [9]. Interestingly in this respect, SEM of the surfaces of bone biopsies, including of human origin, shows that OCs may generate resorption cavities of different shapes [10–12]. More specifically, SEM led to distinguish so-called longitudinally resorption lacunae reflecting long lasting resorption events and reticulate

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[1] OCs, osteoclasts; OC, osteoclast; CatK, cathepsin K; PTH, parathyroid hormone; SEM, scanning electron microscopy; DMSO, dimethyl sulfoxide; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor kappa-B ligand; ES, eroded surface; DC-STAMP, dendritic cell-specific transmembrane protein; TRACP, tartrate resistant acid phosphatase; ClC7; chloride channel 7; NFATc1, Nuclear factor of activated T-cells cytoplasmic 1.

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patch resorption lacunae reflecting several short episodes of intermittent resorption. Furthermore, mathematical models showed that changes in the geometry of single resorption cavities are already sufficient to affect bone stiffness [13]. Taken together, these observations suggest that attention should be paid on the mechanism directing where exactly the OC resorbs bone, in addition to the mechanism controlling how much bone the OC is removing.

OC resorption patterns and their response to different treatments have primarily been analyzed in cultures of OCs on bone slices [14,15]. When cultured alone, most OCs typically excavate bone to a certain depth, then stop and migrate to a new resorption site, thereby generating a series of discrete round excavations often next to each other, which thus reflect intermittent resorption. Addition of estrogen to these osteoclast cultures, induces shallower excavations [16], whereas addition of glucocorticoids induces continuous resorption trenches instead of round discrete excavations, meaning that resorption tends to keep on going over an extended length without interruption by migration episodes [17]. But what is the mechanism determining these respective resorption behaviors? Interestingly, SEM shows that demineralized collagen is present at the bottom of the round excavations generated in control conditions, as well as in the shallower ones generated in the presence of estrogen, but not in the elongated trenches induced by glucocorticoids [16,17]. This shows that OCs in control and estrogen conditions solubilize collagen more slowly than mineral, and that agents stimulating the rate of collagenolysis relative to that of demineralization, such as glucocorticoids, may prolong the duration of resorption events. We therefore hypothesized that the balance between the rate of collagenolysis and demineralization might serve as a mechanism determining the duration of a resorption event, and thereby also the excavation geometry.

A definitive demonstration of this hypothesis requires testing the effect of direct and specific inhibitors of either mineral solubilization or collagen degradation, on the resorption pattern of OCs. We used inhibitors of CatK to slow down the relative rate of collagen degradation compared to the rate of mineral solubilization [18–20], and we used low concentrations of a carbonic anhydrase inhibitor to increase the relative rate of collagen degradation compared to mineral solubilization [21]. Thus, as illustrated in Fig. 1, according to our hypothesis, CatK inhibitors should accelerate the accumulation of collagen in the resorption pit thereby leading to early termination of the local resorption event and a shallower pit. In contrast, mild inhibition of carbonic anhydrase should allow collagenolysis to proceed as fast as demineralization, thereby ensuring continuation of the local resorption event, thus promoting the formation of trenches at the expense of round pits.

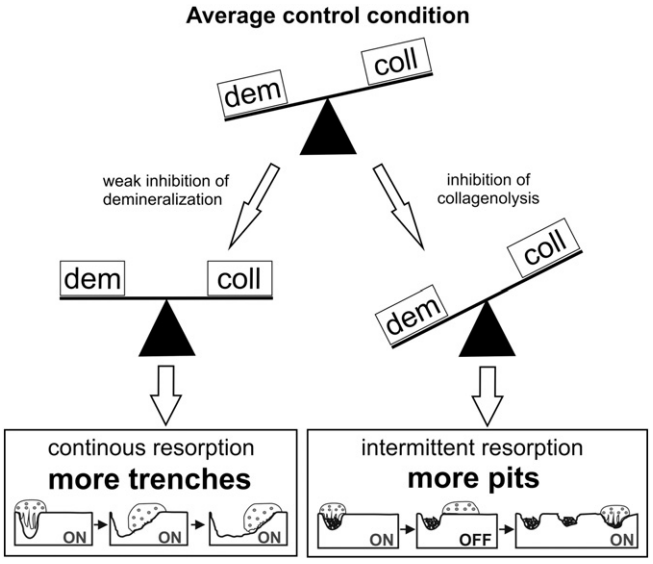

Fig. 1. Experimental approach for testing whether the relative rate of collagenolysis and demineralization affects the duration of resorption events and their geometry. In average control conditions OCs seeded on bone slices show a rate of collagenolysis (coll) which is slower than the rate of demineralization (dem). Inhibition of collagenolysis will result in an even greater imbalance between these two rates, thereby leading to a faster accumulation of collagen. Our hypothesis predicts that pits will then be shallower and resorption behavior more intermittent, in accordance with early termination of every resorption event. In contrast, slowing down the rate of demineralization will allow collagenolysis to proceed as fast as demineralization, so that collagen will no longer accumulate in the excavations thereby allowing continuous contact with mineral. Our hypothesis predicts that most resorption events will then appear as long trenches, which reflect continuous resorption.

Materials and methods

Inhibitors of OC resorption

The following inhibitors of OC resorption were used: 6-ethoxyzolamide (Sigma-Aldrich, Broendby, Denmark), specific inhibitor of carbonic anhydrase, 20 mM stock in DMSO, stored at −20 °C; E64 (Sigma-Aldrich), cysteine-protease inhibitor, 1 mM stock in H_2O, stored at −20 °C; L873724, an inhibitor specific of CatK [20,22,23] (a generous gift from MSD, Rahway, USA), 10 mM stock in DMSO (Sigma-Aldrich) stored at −20 °C.

OC resorption assay

Human $CD14^+$ cells were isolated from buffy coats of healthy volunteers (approved by the local ethics committee, 2007-0019) and differentiated into multinucleated OCs through the use of 25 ng/ml M-CSF and 25 ng/ml RANKL (R&D, Abingdon, England, UK) as described previously [17]. Differentiated OCs were re-seeded on bovine cortical bone slices adapted for 96-well plates (IDS Nordic, Herlev, Denmark) at a density of 50,000 to 100,000 cells per bone slice, and cultured for 72 h in the presence or not of various resorption inhibitors at the indicated concentrations. DMSO was added at a final concentration of 0.2% to controls when relevant.

The resorption features (i.e. cavitations as well as superficial demineralization patches) were stained with toluidine blue as described previously [17] and analyzed through light microscopy. Resorbed bone surface area, number of resorption cavities and maximal erosion depth measurements were measured as previously described [17]. A resorption feature with a continuous and distinct perimeter at the surface was counted as one. The resorption cavities were sub-divided into: 1) round excavations which were termed “pits” and 2) elongated excavations appearing as continuous grooves which were termed “trenches”. The latter were at least twice as long as wide.

Removal of organic matrix from resorption lacunae and rendering bone slices inorganic

In order to determine the thickness of the collagen layer in the resorption lacunae, maximum resorption depths were measured before and after treatment with NaOCl and the difference between these two depth measurements was calculated as an assessment of the thickness of the collagen layer, as previously described [17].

Removal of organic matrix prior to seeding of OCs for resorption was performed in a similar fashion. Each bone slice was transferred into 500 μl 5–7% NaOCl and incubated at room temperature for 15 min while shaking in a thermomixer. Subsequently, the bone slices were washed individually in 50 ml sterile ddH_2O while shaking for 30 min. The bone slices were stored for up to a week in sterile ddH_2O at 4 °C until use.

Analysis of gene expression

Differentiated OCs were obtained as explained above. The cells were lyzed, RNA purified, cDNA generated and TaqMan Q-RT-PCR